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Clinicopathological roles of Vasobin-2 in colorectal cancers

Prognostic effect of Vasohibin-2

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Abstract

Aim: Vasohibin-2(VASH2) is a pro-angiogenic molecule synthesized from mononuclear cells. The biological characteristics of colorectal cancer (CRC) cells and their microenvironment are not known yet. In the present study, the purpose was to investigate the clinicopathological roles of VASH2 in colorectal cancers.

Material and Methods: Three-micron sections were made for Immunohistochemistry (IHC) Analysis of the paraffin block tissues of 159 patients who underwent curative surgery for CRC. Immunohistochemical staining was performed with anti-CD34 and anti-D2-40' and anti-Vasohibin-2 antibodies as lymphatic vessel markers on vascular endothelial cells. The density of newly formed vessels in the peripheral stroma of the tumor with CD34 and D240 and the presence of VASH-2 were investigated in these vessels.

Results: It was determined in the relationship analysis of the variables that VASH2 positivity showed a positive relationship with tumor diameter ($p<0.05$). No significant relationships were detected with other prognostic factors. Advanced age, perineural invasion (PNI), and pathological stage were the parameters that predicted survival in the Cox Regression Analysis, in which many variables were included ($p<0.05$), and VASH2 positivity showed negative predictive characteristics together with CD 34 positivity ($p<0.05$). No relationship was detected between VASH2 expression levels and CD34 and D-240 in cancer stroma and paracancerous tissue. VASH2 expression was significantly lower in cancer stroma and VASH2, CD34, and D-240 levels were higher in paracancerous tissue; however, no relationship was detected in this respect.

Discussion: In the present study, a significant relationship was detected between VASH2 and tumor diameter. However, no statistically significant differences associated with prognosis were detected. Further studies of its other roles in the tumor microenvironment as well as the pro-angiogenic characteristics of VASH2 will help to reveal the effects of this molecule in cancer angiogenesis.

Keywords

Angiogenesis Stimulating Agents, Endothelial Cells, Immunohistochemistry, Vasohibin

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Introduction

Colorectal cancer is the third most common cancer on a worldwide scale and is the second most common cause of cancer-related mortality [1]. Several hereditary and somatic mutations play roles in its development. It is generally diagnosed at an advanced stage, and only 10% of newly diagnosed cases are in the early stage [2]. In the past decade, colorectal cancer-related mortality has increased in all age groups [3]. It is estimated that 25% of newly diagnosed rectal cancers and 10-12% of colon cancers will be diagnosed under the age of 50 in the next decade [4]. Recurrence occurs in 30-50% of patients after curative treatments, and survival rates decrease in this group [5]. Its prognosis is determined according to the TNM Staging System that was established by the Union for International Cancer Control (UICC) and the American Joint Committee on Cancer (AJCC). Aside from the stage of the disease, other parameters such as biological behavior of the tumor, biomarkers, neoadjuvant or adjuvant oncological treatment preferences, and various risk factors play a role in survival. Histological characteristics such as tumor budding, perineural and lymphovascular invasion, apical lymph node invasion, lymph node involvement rate, molecular markers such as BRAF, MSI, KRAS, CDX2, and the compliance of patients with the treatment play roles in the process.

Angiogenesis is the process of the regeneration of the blood vessels in the pre-existing vascular bed, which takes place physiologically during wound healing, reproduction, and development. It is pathological in the formation of cancer. Neovascularization is required for metastasis and tumor growth. Tumors develop varying degrees of angiogenic phenotypes depending on genetic background and catalysis of the local factors such as hypoxia. The balance between oncogenes and tumor suppressor genes determines the direction of the angiogenesis process. The regulation of angiogenesis occurs between tumor cells, endothelial cells, stromal cells, and inflammatory cells with the help of inhibitory/stimulatory factors [6]. Folkman was the first author to describe and show the presence of a proangiogenic factor [7]. Many proangiogenic and antiangiogenic factors have been described so far, the most prominent among which is Vascular Endothelial Growth Factor, which is induced by hypoxia and various growth factors and cytokines (e.g., EGF, PDGF, TNF-α, TGFβ, interleukin-1β). VEGF, on the other hand, increases vascular permeability and endothelial cell proliferation inducing tube formation and providing secretion of proteolytic enzymes (e.g., plasminogen activator receptor, urokinase, uPA, and MMP1) from vascular endothelial cells. It is effective in the regulation of vascular blood flow with its effects on nitric oxide release. The lymphogenesis process is regulated by the VEGF family. Previous experimental studies have shown that VEGF-C and VEGF-D increase tumor lymphangiogenesis and metastasis process over the lymphatic pathway [8,9]. Secreted by endothelial cells as a result of VEGF stimulus, the Vasohibin 1 (VASH1) molecule, which was described by Sato, also shows antiangiogenesis activity [10]. As another member of the same family and synthesized by bone marrow-derived infiltrative mononuclear cells, Vasohibin 2 (VASH2) stimulates angiogenesis and is also involved in the regulation of the synthesis of FGF 2 and VEGF, which are the main stimulators of VASH1 production. The hypoxic environment results in the expression of hypoxia-inducible Factor 1a via VEGF secreted by all tumor cells. Hypoxia reduces the inducible effects of VEGF on Vasohibin. In vivo studies showed that the Vasohibin family plays roles in many cancer types concerning microvessel density, histological grade, vascular invasion, poor clinical characteristics, metastasis, and spread in the abdominal cavity [11].

In the group of colorectal cancer patients included in the current study, the purpose was to investigate the effectiveness of the VASH2 Family in terms of tumor size, disease stage, metastasis, tumoral histological characteristics, survival, and prognosis.

Material and Methods

The study included 192 patients who underwent colorectal resection because of colon and rectal adenocarcinoma in Tekirdağ Namık Kemal University Medical Faculty Hospital General Surgery Clinic between 2013 and 2021. Clinical and follow-up data of the patients were obtained by retrospective scanning of data files. The data on the age, gender, tumor diameter, presence of metastases, and life expectancy of the patients were obtained from the files. The pathology preparations of these 192 patients were removed from the archives and evaluated again by the pathologist using an Olympus Bx46 brand Light Microscope, and appropriate tumor blocks without necrosis and bleeding were determined. Tumor blocks of 159 patients were retrieved from the pathology block archives and included in the study.

Immunohistochemistry

Three-micron sections were made from paraffin block tissues of these 159 patients for immunohistochemistry analysis. The sections were taken on slides with a positive charge and were deparaffinized with xylol for 15 minutes after waiting for one hour in an oven at 60°C. They were passed through decreasing alcohols and washed in distilled water for hydration. The slides were then placed in Benchmark XT brand device and anti-CD34 (Roche, QBEnd/10, USA) staining was made for vascular endothelial cells, anti-D2-40 (Roche, Mouse Monoclonal Antibody, USA) was used as lymphatic vessel marker, and anti-Vasohibin-2 (Merck, Clone 5E3 Mouse Monoclonal, Japan) was used as immunohistochemical staining method.

Immunohistochemistry evaluation

The slides were evaluated by the pathologist researcher by using a Bx46 Olympus brand Light Microscope, without knowing the clinical course of the patients. The areas where colon cancer cells came into contact with or penetrated the stroma were identified carefully. In the first step, microvessels were counted after scanning the immunostained area by looking for CD34-positive signals at x40 magnification under the light microscope. Locations with the most prominent microvessels were selected. Microvessel Density (MVD) was evaluated with Light Microscopy at×200 magnification in invasive tumor areas that contained the largest number of capillaries and small venules (neovascular spots) per 1 mm2 area according to the original method. The investigation of Lymphatic Vessel Density (LVD) was performed by using the same procedure described above, searching for D2-40-positive signals. Then, immunoreactive ratios were evaluated for Vasohibin2 in tumor

peri-stroma microvessels and tumoral cells (Figure 1). The density of newly formed vessels in the peripheral stroma of the tumor with CD34 and D240 and the presence of Vasohibin2 were investigated in these vessels. Vasohibin positivity was noted in neoplastic cells in some of the cases, as seen in (E).

Statistical analysis

The distribution of numerical data was analyzed with the Shapiro-Wilk test. While Pearson's test was used for the relationship between parametric data, the Spearman Test was preferred for data showing heterogeneous distribution. The Kaplan-Meier method was used for survival curves, and the survival effect of the variables was calculated with the proportional hazards model. A p-value below 0.05 was accepted for statistical significance. All analyzes were performed with SPSS v 22 for windows software.

Ethics committee approval:

This study was approved by Tekirdağ Namık Kemal University Health Research Ethics Committee (No: 2021.123.04.18)

Results

Among the patients, 73 (38%) were female and 119 (62%) were male. The mean age was 65 years (23-89), mean tumor diameter was 49.4 mm (1-170). The majority of the patients were recto-sigmoid cancers (n:108 - 56.2%). Tumor localizations are summarized in Figure 2.

Table 1. Relationship betweenVasohibin 2 positivity and other variables.

Variables	Relationship Coefficient	P -value
Age	0.009	0.915
Histological grade	0.019	0.816
Tumor diameter	0.198	0.013
Metastatic lymph nodes	-0.116	0.151
CD 34 positivity	-0.019	0.818
D 240 positivity	0.075	0.350
Pathological Stage	-0.107	0.185
Neutrophil	0.039	0.632
Lymphocyte	-0.018	0.828

Table 2. Predictive values of variables on survival.

Variables	B	P -v-alue	Exp(B)
Age	0.045	0.002	1.046
Histological grade	0.106	0.748	1.111
LVI	0.148	0.672	1.159
PNI	0.670	0.037	0.512
Tumor diameter	-0.002	0.801	0.998
Metastatic lymph nodes	0.130	0.073	1.139
Vasohibin 2	-0.140	0.046	0.870
CD 34 positivity	-0.049	0.008	0.953
D 240 positivity	0.011	0.788	1.011
Pathological Stage	0.236	0.006	1.267
Localization	0.171	0.598	1.186
Neutrophil	0.016	0.886	0.984
Lymphocyte	0.172	0.643	0.842

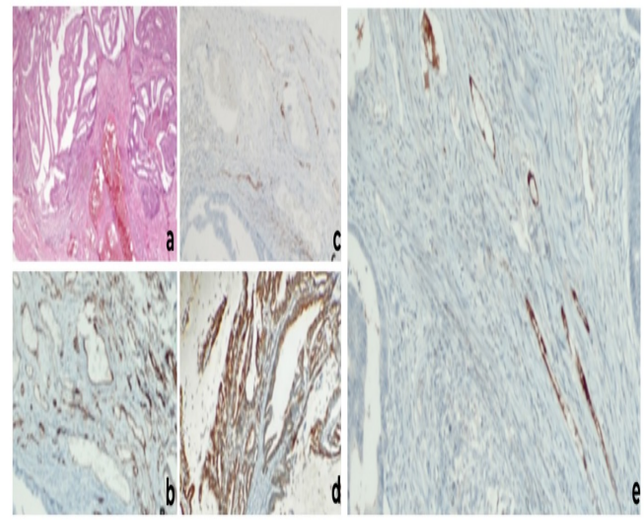

Figure 1. (A) H&E staining and immunostaining for (B) CD34, (C) D240, (D) and (E) VASH2.

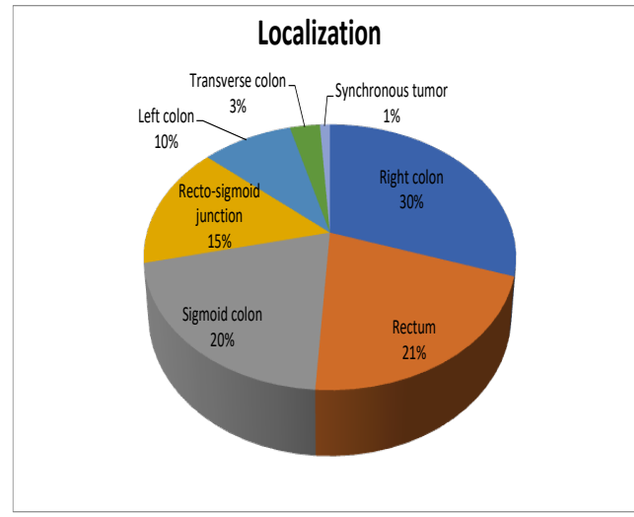

Figure 2. Tumor localization distributions.

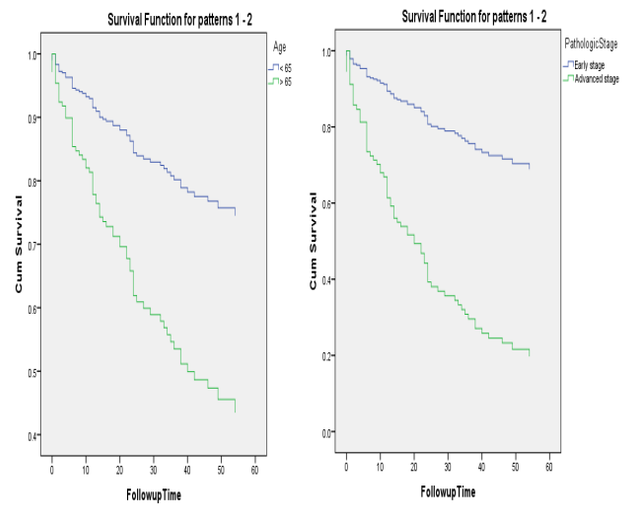

Figure 3. Survival curves for age and clinical stage.

The number of patients who were metastatic at the time of diagnosis was found to be 25 (13%). Stage 3, Stage 2 and Stage 1 patients were 73 (38%), 59 (30.7%), and 35 (18.2%), respectively. All patients underwent curative surgery, however, 6 (3.1%) patients received hyperthermic intraperitoneal chemotherapy in addition to cytoreductive surgery (hipec). Grade 2 patients were in the majority (133-69.3%) in the postoperative histological examination. Although 48 (25%) patients had Grade 1 tumors, 11 (5.7%) patients had Grade 3 tumors. The LVI rate was 56.8% (109) and the PNI rate was 31.3% (60). A total of 123 (64%) of the patients survived as of 01.11.2022 and 69 (36%) of them died. The mean follow-up period was calculated as 32 (0-77) months. A mean of 16 (3-44) CD 34+ and a mean of 5 (1-20) D240+ vascular structures were counted at 40 magnification in the re-examination performed in 159 patients. VASH2 was not expressed in 18 (11.5%) patients. The mean number of VASH2-stained vascular structures was 3 (0-20).

In the relationship analysis of the variables, it was determined that VASH2 positivity showed a positive relationship with tumor diameter ($p<0.05$ cc: 0.198). However, no significant relationship was detected with prognostic factors such as age, stage, PNI, and LVI (Table 1).

Advanced age, PNI, and pathological stage were the parameters that predicted survival in the Cox Regression Analysis, in which many variables were included ($p<0.05$). VASH2 positivity showed negative predictive characteristics together with CD 34 positivity ($p<0.05$) (Table 2).

The cut-off value was calculated as 65.5 in the ROC curve drawn according to age (AOC: 0.631). The cut-off value for the clinical stage was determined as Stage 3C (AOC: 0.707). The age and clinical stage-dependent survival curves are summarized in Figure 3.

Discussion

VASH2 induces angiogenesis by triggering increased VEGF levels over HIF 1α under hypoxic conditions [10]. It consists of 355 amino acids, is 55% similar to VASH1, and is found in mononuclear cells rather than endothelial cells [11]. It can stimulate tumor cells to proliferate, migrate and infiltrate. With their decreased levels, the abnormality in the vascular structure improves, and tumor growth is suppressed. It also reduces p53 levels and shows anti-apoptosis activity by reducing Bax Expression [12,13]. In the study that was conducted by Liu et al., it was emphasized that the level of VASH1, which is another member of the same family, with antiangiogenetic characteristics, exhibited a negative relationship with tumor size and a positive relationship with the presence of advanced stage and distant metastases in colon cancer patients [14]. In the study conducted by Du et al., aside from the role they play in angiogenesis, it was reported to be effective in microvessel density, histological grade of tumor, level of invasion, poor clinical outcomes, rate of metastasis, and extent of tumor spread within the abdominal cavity [12]. VASH2 is mainly expressed in cancer cells and has a prognostic significance for most cancer types [15,16,17]. However, the evaluation of plasma concentrations of VASH1 and VASH2 is at low levels. It was considered that it would be beneficial to conduct a study to elucidate whether their routine use would be beneficial in clinical practice. In light of this information, in the present study, the interaction between VASH2 and tumor size, grade level, number of metastatic lymph nodes, lymphovascular and perineural invasion level, clinical stage, relationship with age and gender, tumor localization, and survival were investigated in patients who were diagnosed with colorectal cancer and whose treatment process was completed. Positive relationships were detected between tumor size and VASH2, which showed angiogenic activity ($p=0.013$). No significant relationships were detected between histological grade level ($p=0.816$), metastatic lymph node number ($p=0.151$), pathological stage ($p=0.185$), and other parameters. Although the rate of lymphovascular and perineural invasion of the tumor (CD 34 and D2-40 positivity rate) decreased with increasing age, it was remarkable that there were no significant changes in VASH2 levels. Gender differences did not cause any differences in VASH2 levels ($p=0.730$). The positive relationship between VASH2 and tumor size contributed to the literature data. No relationship was detected between the presence of the tumor in the colon or rectum and different localizations in the colon and the VASH2 levels ($p=0.59$). Considering the VASH2 angiogenesis activity, the expected relationship between lymphovascular invasion and the number of metastatic lymph nodes was not found to be significant in the current study. Also, no relationship was detected between VASH2 expression levels in cancer stroma and cancer tissue and CD34 and D-240. VASH2 expression was significantly lower in cancer stroma. VASH2, CD34, and D-240 levels were higher in the tissue around cancer, but no relationship was detected between VASH2, CD34, and D-240.

Takahashi et al. showed that, in serousovarian cancer, VASH2 expression is a poor prognostic factor [15]. It is already known that VASH2 is involved in the epithelial-mesenchymal transition because of regulating TGF-β signaling. It also plays important roles in tumor progression through stromal activation of cancer-associated fibroblasts [16]. As well as its pro-angiogenic activity, it probably has various other roles in the tumor microenvironment. The relationship between Vasohibin expression and disease prognosis was investigated in the pancreatic, liver, breast, and many other cancer types and has been shown in many previous studies [17-19]. It was suggested that VASH2 expression induces angiogenesis, and therefore, contributes to tumor growth [20,21]. It was observed in the current study that age, perineural invasion, and pathological stage were associated with poor prognosis, but not with VASH2 expression. Similarly, contrary to expectations, no relationship was detected between the stage of the disease and the metastatic disease.

The first limitation of the study was that it had a retrospective design. A total of 25 (13%) patients had metastases (Stage 4) at the time of diagnosis in the group, and 70% of the patients were histopathologically classified as Grade 2. Intra-abdominal spread rates were not assessed. Conditions such as abdominal aortic aneurysm, peripheral vascular disease, atherosclerosis, age-related macular degeneration, association with other malignancies (ovary, esophagus, pancreas, lung, stomach, breast, hepatocellular Ca, etc.), renal functions, which were shown to be associated with the Vasohibin family, could not be

studied in the patient group [22-25]. Conducting a prospective study in a more homogeneous patient group will shed light on the questions that must be answered.

The present study showed that VASH2 has a significant relationship with tumor diameter, but not with other prognostic factors. To understand the accuracy of this, prospective studies on homogeneous patient groups with similar tumor stages are needed.

Conclusion

The expression of VASH2 in the cancer cell, cancer stroma, and the area around the cancerous tissue causes a prognostic difference. Effective studies are needed to understand the factors affectingthe expression and roles they play in the microenvironment. In this way, it will be understood whether it is a predictive factor for tumor prognosis in the future.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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Conflict of interest

None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.

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